Human rotavirus strains bearing VP4 gene P[6] allele recovered from asymptomatic or symptomatic infections share similar, if not identical, VP4 neutralization specificities

Yasutaka Hoshino,[a],* Ronald W. Jones,[a] Jerri Ross,[a] Norma Santos,[b] and Albert Z. Kapikian[a]

[a] *Epidemiology Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892-8026, USA*
[b] *Departamento de Virologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Abstract

A rotavirus VP4 gene P[6] allele has been documented in a number of countries to be characteristically associated with an endemic predominantly asymptomatic infection in neonates in maternity hospital nurseries. The mechanisms underlying the endemicity and asymptomatic nature of such neonatal infections remain unknown. Rotavirus strains sharing this same P genotype, however, have more recently been recovered from an increasing number of symptomatic diarrheal episodes in infants and young children in various parts of the world. Previously, we have shown that an asymptomatic P[6] rotavirus neonatal infection is not associated with a unique VP7 (G) serotype but may occur in conjunction with various G types. Although amino acid sequence comparisons of the VP4 gene between selected "asymptomatic" and "symptomatic" P[6] rotavirus strains have been reported and yielded information concerning their VP4 genotypes, serotypic comparisons of the outer capsid spike protein VP4 of such viruses have not been studied systematically by two-way cross-neutralizations. We determined the VP4 neutralization specificities of four asymptomatic and four symptomatic P[6] strains: two each of asymptomatic and symptomatic strains by two-way tests, and two each of additional asymptomatic and symptomatic strains by one-way tests. Both asymptomatic and symptomatic P[6] strains were shown to bear similar, if not identical, VP4 neutralization specificities. Thus, P[6] rotavirus strains causing asymptomatic or symptomatic infections did not appear to belong to unique P (VP4) serotypes. In addition, a close VP4 serotypic relationship between human P[6] rotavirus strains and the porcine P[6] rotavirus Gottfried strain was confirmed.
© 2003 Elsevier Inc. All rights reserved.

Introduction

Group A rotaviruses remain the single most important etiologic agents of severe diarrhea in infants and young children worldwide. Rotavirus disease is estimated to be responsible for approximately up to 520,000 deaths each year among children < 5 years of age predominantly in developing countries (Miller and McCann, 2000). In the United States, it is estimated that approximately 2.7 million infants and young children develop rotavirus diarrhea annually, resulting in approximately 20 deaths, about 50,000 hospitalizations, and more than 500,000 medical visits at an annual societal cost of more than \$1 billion (Bresee et al., 1999; Glass et al., 1994; Kapikian et al., 2001). Thus, the availability of a safe and effective rotavirus vaccine capable of preventing this enormous health burden would represent a global public health breakthrough.

In 1975, only 2 years after first being identified in humans, rotaviruses were detected in a number of countries in the stools of predominantly asymptomatic neonates in newborn nurseries (Albrey and Murphy, 1976; Bishop et al., 1976; Chrystie et al., 1975; Madeley et al., 1978; Murphy et al., 1975; Totterdell et al, 1976). These early reports as well as reports published later (Bryden et al., 1982; Cicirello et al., 1994; Das et al., 1994; Garbag-Chenon et al., 1985; Grillner et al., 1985; Haffejee et al., 1990; Jayashree et al., 1988; Kilgore et al., 1996; Linhares et al., 2002; Perez-

* Corresponding author. National Institutes of Health, Building 50, Room 6308, 50 South Drive MSC 8026, Bethesda, MD 20892-8026. Fax: +1-301-480-1387.
E-mail address: THOSHINO@niaid.nih.gov (Y. Hoshino).

Schael et al., 1984; Spencer et al., 1988; Steele and Alexander, 1987; Steele et al., 2002; Sukumaran et al., 1992; Tam et al., 1990; Tietzova et al., 1995; Tufvesson et al., 1986) showed that (i) a majority of the infected newborn babies were characteristically symptom free or only minimally symptomatic and (ii) a single rotavirus strain with a distinct electropherotype persisted in a neonatal nursery for a long period of time. The latter observation was in sharp contrast to rotavirus strains circulating in the surrounding community, that demonstrated a high degree of genomic variability. Previously, selected "endemic nursery" rotavirus strains recovered from asymptomatic neonates in four different countries (Australia, Sweden, the United Kingdom, and Venezuela) were analyzed by (i) neutralization, (ii) RNA–RNA hybridization, or (iii) VP4 gene sequence analysis. It was reported that (i) there was no correlation between a specific G serotype of rotavirus and the occurrence of asymptomatic or mild infections among newborn babies (Hoshino et al., 1985) and (ii) the VP4 genes, which were the only conserved genes among the four neonatal rotavirus strains tested as determined by RNA–RNA hybridization (Flores et al., 1986), were unique to each of these nursery strains and were closely related to each other (95.5 to 97.5% nucleotide identity) (Gorziglia et al., 1988, 1990). Because of (i) the possible association between this distinct VP4 gene allele (P2A[6]) and the observed attenuation phenotype in neonates and (ii) an observation reporting that an asymptomatic or mild infection of neonates with one of such endemic nursery rotavirus strains conferred protection against clinically severe disease due to reinfection during the first 3 years of life (Bishop et al., 1983), two such P[6] rotavirus strains were developed independently as candidate vaccines. One (M37) was of Venezuelan origin and the other (RV3) of Australian origin, and each underwent phase I to early phase II clinical trials (Barnes et al., 1997, 2002; Flores et al., 1990; Midthun et al., 1991; Perez-Schael et al., 1994; Vesikari et al., 1991). Moreover, RV3 is still under study as a vaccine candidate (Barnes et al., 2002). Of note was the finding that the VP4 of the porcine rotavirus Gottfried strain which was originally isolated from a diarrheic pig in the U.S.A. (Bohl et al., 1984) was closely related genetically (the Gottfried VP4 shares 87.7–89.3% amino acid identity with asymptomatic P[6] VP4s) and antigenically to human nursery strains with P2A[6] specificity and therefore was classified as a subtype (P2B[6]) (Li and Gorziglia, 1993).

Recent epidemiologic surveys of rotavirus strains have reported that strains bearing the VP4 gene P[6] allele in conjunction with various G types are recovered from an increasing number of infants and young children with diarrhea throughout the world (for reviews, see Cunliffe et al., 2001; Gentsch et al., 1996; Hoshino and Kapikian, 2000). Such observations have aroused renewed scientific interest as to whether any differences exist in the genetic make-up between "asymptomatic" and "symptomatic" P[6] rotavirus strains. Efforts have been made, therefore, to seek a corre-

lation between amino acid sequence differences of selected genes [which included VP4 (or VP8*), VP7, NSP1, or NSP4 gene, each of which had been reported in an animal model to be virulence-associated (Bridger et al., 1998; Broome et al., 1993; Hoshino et al., 1995; Offit et al., 1986)], of asymptomatic or symptomatic P[6] rotavirus strains and the observed differences in virulence phenotype (Kirkwood et al., 1996; Lee et al., 2000, 2001; Pager et al., 2000; Palombo and Bishop, 1994; Santos et al., 1994). However, appreciable amino acid differences in such selected genes have not been identified consistently thus far. Although one-way neutralization tests have been reported for four asymptomatic strains (M37, 1076, McN, and ST3) (Gorziglia et al., 1990) and two symptomatic strains (MW23 and US1205) (Cunliffe et al., 2000; Kirkwood et al., 1999) and were consistent with the observed genotypes, it is still not known if they are identical by reciprocal neutralizations tests. The objectives of this study were threefold, as follows: (i) to determine the reciprocal two-way neutralization specificity of the outer capsid spike protein VP4 of two asymptomatic (M37 and ST3) and two symptomatic (MW23 and US1205) P[6] strains; (ii) to determine the one-way neutralization specificity of two additional asymptomatic (1076 and McN) and two additional symptomatic (INL1 and R143) strains; and (iii) to determine the extent of the subtype relationship of the porcine rotavirus Gottfried strain VP4 by neutralization with these asymptomatic and symptomatic strains.

Results

Generation of single VP7 or VP4 gene substitution human × human, human × bovine, or porcine × human rotavirus reassortants as well as hyperimmune guinea pig antiserum to each reassortant

We generated (i) two single VP7 gene substitution human × human rotavirus reassortants, each of which had 10 genes from human P2A[6] rotavirus M37 or ST3 strain and only the VP7 gene from human rotavirus DS-1 strain (G2); (ii) three single VP4 gene substitution human × bovine rotavirus reassortants, each of which had only the VP4 gene of human P2A[6] rotavirus M37, MW23, or US1205 strain and the remaining 10 genes of bovine rotavirus UK strain (G6); and (iii) one single VP7 gene substitution porcine × human rotavirus reassortant which had 10 genes from porcine rotavirus Gottfried strain (P2B[6],G4) and only the VP7 gene from DS-1 strain (Fig. 1). Reassortants M37 × DS-1 (P2A[6],G2) and M37 × UK (P2A[6],G6) were constructed to analyze whether a different VP7 gene background (DS-1 or UK) affects qualitatively or quantitatively the immunologic and biologic properties of the M37 VP4 protein of the reassortant because of a "fit" difference (Chen et al., 1992). We have chosen strains DS-1 and UK to generate various reassortants because (i) VP7 of the DS-1 or
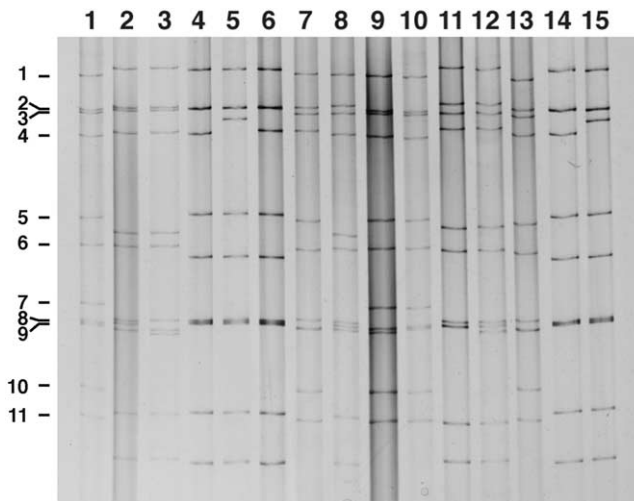
Fig. 1. Electrophoretic migration patterns of genomic RNAs of human rotavirus DS-1 strain (lane 1), reassortant M37 × DS-1 (lane 2), human rotavirus M37 strain (lane 3), reassortant M37 × UK (lane 4), bovine rotavirus UK strain (lane 5), reassortant MW23 × UK (lane 6), human rotavirus MW23 strain (lane 7), human rotavirus ST3 strain (lane 8), reassortant ST3 × DS-1 (lane 9), human rotavirus DS-1 strain (lane 10), reassortant Gottfried × DS-1 (lane 11), porcine rotavirus Gottfried strain (lane 12), human rotavirus US1205 strain (lane 13), reassortant US1205 × UK (lane 14), and bovine rotavirus UK strain (lane 15) in 10% polyacrylamide gel. Genomic RNAs were electrophoresed at 13 mA for 15 h and the resulting migration patterns were visualized by staining of gel with silver nitrate.

UK is the least cross-reactive by neutralization and thus the VP4 and VP7 responses are readily dissociated and (ii) various single VP7 (DS-1 or UK) substitution reassortants had been used extensively by us in characterizing VP4 neutralization specificities of various rotavirus strains (Hoshino and Kapikian, 1996). Hyperimmune antiserum to each of the six reassortants was generated by immunizing guinea pigs which were free of rotavirus antibodies by neutralization (antibody titer < 1:20 versus M37). At least two guinea pigs were used for immunization against each reassortant in an attempt to obtain an accurate neutralization profile.

Analysis of neutralization profile of guinea pig hyperimmune antiserum raised against VP4 protein of human or porcine P[6] rotavirus strains

Table 1 summarizes the antigenic characterization of human or porcine P2A[6], P2B[6], or P?[6] rotavirus VP4s. Previously, hyperimmune guinea pig antiserum raised against asymptomatic 1076 VP4 expressed by a baculovirus recombinant was reported to neutralize selected asymptomatic endemic nursery strains M37, McN, and ST3 (Gorziglia et al., 1990). We confirmed and extended this observation by showing the existence of a two-way VP4 antigenic relationship among the 1076, M37, and ST3 strains (Table 1). Similarly, by one-way cross-neutralization, symptomatic

strains MW23 and US1205 were previously shown to be neutralized significantly by hyperimmune guinea pig antiserum to ST3 (asymptomatic P2A[6] strain) × DS-1 (Cunliffe et al., 2000; Kirkwood et al., 1999). This finding was confirmed and extended further in this study by two-way cross-neutralization: antibodies to the MW23 VP4 or US1205 VP4 neutralized significantly all four asymptomatic strains including the ST3 virus. Similarly, the asymptomatic M37 VP4 was shown to be related antigenically in a two-way fashion to the symptomatic MW23 or US1205 VP4. Furthermore, two additional symptomatic P?[6] strains examined (Indian INL1 strain and Brazilian R143 strain) were neutralized significantly by antibodies to asymptomatic (M37 and ST3) or symptomatic (MW23 and US1205) VP4s and thus could be classified as P2A strains. Thus, this study showed that both asymptomatic and symptomatic P[6] strains bore similar, if not identical, neutralization specificities. Hyperimmune guinea pig antiserum raised against M37 × DS-1 or M37 × UK demonstrated a similar neutralization profile against both asymptomatic and symptomatic P[6] strains (Table 1), indicating that the different genetic background (DS-1 or UK) of each reassortant did not affect qualitatively or quantitatively the immunologic and biologic characteristic of the M37 VP4.

All eight guinea pig hyperimmune antisera raised to the VP4 protein of asymptomatic or symptomatic human P2A[6] or P?[6] rotavirus strains neutralized the porcine rotavirus Gottfried strain (P2B[6]) efficiently with titers ranging from identical to eight-fold less than to the human homotypic P2A[6] or P?[6] rotavirus strains, indicating that both human and porcine P[6] rotavirus VP4s carried similar neutralization specificities. These results are in accord with those reported previously by Gorziglia et al. (1990). However, antibodies to the Gottfried VP4 neutralized human P2A[6] or P?[6] strains 8- to 16-fold less efficiently than the Gottfried virus (Table 1), indicating that in this direction the VP4 relationship, although within 20-fold of the Gottfried virus by neutralization (i.e., 20 antibody units, wherein 1 unit equals the least amount needed to produce the response being studied) (Hoshino and Kapikian, 1996), was not as close as that noted above. In addition, although our results are also consistent with a designation of Gottfried as a subtype of P2A, i.e., as P2B, as suggested later by Li and Gorziglia, our neutralization findings differ from the latter report in which guinea pig serum raised to recombinant Gottfried VP4 neutralized asymptomatic human P2A[6] neonatal viruses within 20 antibody units, whereas guinea pig serum to a recombinant asymptomatic human neonatal 1076 strain VP4 did not neutralize Gottfried virus within 20 antibody units (Li and Gorziglia, 1993). Thus, in that study Gottfried strain had the characteristics of a prime strain when compared to the asymptomatic human strains. In our study as stated above, each of the eight guinea pig sera raised to the established or newly established human P2A[6] strains (four to asymptomatic and four to symptomatic strain reassortants) neutralized Gottfried virus more effi-

Table 1
Antigenic characterization of human or porcine P[6] rotavirus VP4s by analysis of their guinea pig hyperimmune antiserum neutralization profile

Rotavirus						A*/S*	Reciprocal of 60% PRN antibody titer of guinea pig hyperimmune antiserum to indicated rotavirus reassortant*****									
Strain	Country of origin	Year collected	Host	P type [genotype]	G type		M37 × DS-1 (P2A[6],G2) 53084**	M37 × UK (P2A[6],G6) 58742	ST3 × DS-1 (P2A[6],G2) 45063	ST3 × DS-1 (P2A[6],G2) 45064	MW23 × UK (P2A[6],G6) 58640	MW23 × UK (P2A[6],G6) 58641	US1205 × UK (P2A[6],G6) 57674	US1205 × UK (P2A[6],G6) 57675	Gott × DS-1 (P2B[6],G2) 44728	Gott × DS-1 (P2B[6],G2) 44729
M37[a]	Venezuela	1982	Human	2A [6]	1	A	**2560** ***	**10240**	**2560** (1)	**2560** (1)	**5120** (0.5)	**640** (2)	**2560** (1)	**2560** (2)	80 (16)	160 (16)
1076[b]	Sweden	1983	Human	2A [6]	2	A	**40960** *****	**2560** (4)	**10240**	**5120**	**1280** (2)	**320** (4)	**2560** (1)	**2560** (2)	10240	10240
McN[b]	Australia	1980	Human	2A [6]	3	A	**2560** (1)	**2560** (4)	**2560** (1)	**2560** (1)	**2560** (1)	**640** (2)	**10240** (0.25)	**5120** (1)	160 (8)	320 (8)
ST3[c]	UK	1975	Human	2A [6]	4	A	**1280** (2)	**2560** (4)	**2560**	**2560**	**5120** (0.5)	**640** (2)	**5120** (0.5)	**1280** (4)	80 (16)	160 (16)
MW23[d]	Malawi	1997/99	Human	2A [6]	8	S	**2560** (1)	**2560** (4)	**2560** (1)	**640** (4)	**2560**	**1280**	**10240** (0.25)	**10240** (0.5)	160 (8)	320 (8)
US1205[e]	USA	1996/97	Human	2A [6]	9	S	**2560** (1)	**1280** (8)	**640** (4)	**640** (4)	**2560** (1)	**640** (2)	**2560**	**5120**	80 (16)	320 (8)
INL1[f]	India	1994	Human	? [6]	9	S	1280 (2)	2560 (4)	2560 (1)	2560 (1)	2560 (1)	640 (2)	2560 (1)	5120 (1)	160 (8)	320 (8)
R143[g]	Brazil	1999	Human	? [6]	9	S	5120 (0.5)	5120 (2)	5120 (0.5)	5120 (0.5)	5120 (0.5)	1280 (1)	5120 (0.5)	5120 (1)	160 (8)	320 (8)
Gottfried[h]	USA	1983	Porcine	2B [6]	4	S	640 (4)	1280 (8)	2560 (1)	640 (4)	1280 (2)	320 (4)	2560 (1)	2560 (2)	**1280**	**2560**
DS-1[i]	USA	1976	Human	1B [4]	2	S	10240 ****	<80	5120	5120	<80	<80	<80	<80	10240	10240
UK[j]	UK	1975	Bovine	7 [5]	6	S	<80	10240	<80	<80	10240	2560	10240	10240	<80	<80

Note. Since two guinea pig hyperimmune antisera raised against M37 × DS-1 or M37 × UK demonstrated a similar neutralization profile, neutralization data for only one serum/reassortant are shown.

* A = asymptomatic infection; S = symptomatic infection.

**Guinea pig identification number.

***VP4 homotypic values in bold.

****VP7 homotypic values underlined.

*****VP4 and VP7 homotypic values in bold and underlined.

******Number in parentheses after each titer indicates the fold difference in titer relative to the homologous titer.

[a] Perez-Schael et al. (1984).

[b] Hoshino et al. (1985).

[c] Wyatt et al. (1983).

[d] Cunliffe et al. (2000).

[e] Ramachandran et al. (1998).

[f] Ramachandran et al. (2000).

[g] Santos et al. (2001).

[h] Bohl et al. (1984).

[i] Wyatt et al. (1982).

[j] Woode et al. (1975).

ciently than defined for the neutralization of each of these strains by guinea pig sera raised to the Gottfried virus reassortant. We have no conclusive explanation for these conflicting results. However, it is possible that this may have resulted from the different source of antigens (expressed recombinant VP4 proteins versus reassortants rotavirus VP4s) used to generate the antibodies. In addition, the lower titer of guinea pig antiserum raised against recombinant VP4 proteins may have played some role in these conflicting results.

Discussion

Rotavirus infections in newborns continue to attract considerable attention of both clinical and basic research investigators worldwide as evidenced by a large number of publications (e.g., Albrey and Murphy, 1976; Bishop et al., 1976; Bryden et al., 1982; Chrystie et al., 1975; Cicirello et al., 1994; Das et al., 1994; Garbag-Chenon et al., 1985; Grillner et al., 1985; Haffejee et al., 1990; Jayashree et al., 1988; Kilgore et al., 1996; Linhares et al., 2002; Madeley et al., 1978; Murphy et al., 1975; Oelofsen et al., 1985; Perez-Schael et al., 1984; Spencer et al., 1988; Steele and Alexander 1987; Steele et al., 2002; Sukumaran et al., 1992; Tam et al., 1990; Tietzova et al., 1995; Totterdell et al., 1976; Tufvesson et al., 1986). That is because the nature of neonatal rotavirus infections is uniquely different in many respects from that in older infants or children. For example, the majority of rotavirus infections in newborns are characteristically asymptomatic or only minimally symptomatic and usually occur shortly after birth, and in some cases <24 h after birth (Haffejee et al., 1990). Moreover, a majority of such endemic nursery strains have been shown to carry a distinct VP4 gene P[6] allele which had not been detected until recently among circulating community rotavirus strains that were associated with diarrhea in older children. It is astonishing to note that rotavirus strains carrying P1A[8] specificity, which constitutes the majority of field isolates in diarrheal patients worldwide (Gentsch et al., 1996; Hoshino and Kapikian, 2000; Kapikian et al., 2001; Koshimura et al., 2000), have never been documented to cause endemic and predominantly symptom-free infections in neonates. It is also noteworthy that another common VP4 serotype P1B[4] has not been observed to cause endemic and predominantly asymptomatic infections in neonates.

It is still an unsettled issue as to what factors (both host and viral) are responsible for establishing endemic, predominantly asymptomatic, or mild P[6] rotavirus infections in neonates. Earlier studies explored possible host factors involved in the unique P[6] rotavirus-neonate ecosystems such as maternal antibody, soluble and cellular components in breast milk, intestinal bacterial flora, physiologic immaturity of the infant gut, and birth weight of the neonate (for reviews, see Haffejee, 1991; Hoshino et al., 1985, and

references therein). As for viral factors, we previously reported that no correlation existed between the occurrence of asymptomatic or mild infections in neonates and a unique rotavirus VP7 (G) serotype or subgroup (Hoshino et al., 1985). Previously, the VP4 of two symptomatic P[6] strains (MW23 and US1205) were reported to be related antigenically in a one-way fashion to asymptomatic (ST3) VP4. In the present study, we showed by two-way cross-neutralization that both asymptomatic (M37 and ST3) and symptomatic (MW23 and US1205) bore similar, if not identical, VP4 neutalization specificities. In addition, the VP4 of two additional symptomatic strains (INL1 and R143) was shown to be related serotypically in a one-way fashion to asymptomatic (M37 and ST3) VP4s. Thus, an asymptomatic or symptomatic P[6] rotavirus infection was not associated with unique VP4(P) serotypes in the limited number of strains examined in this study. These serological relationships among P[6] VP4s correlate well with molecular relationships among them: strains M37, 1076, McN, ST3 MW23, and US1205 share >94% amino acid identity. In addition, we showed that guinea pig hyperimmune antisera raised against asymptomatic or symptomatic human P2A[6] or P?[6] rotavirus VP4 neutralized porcine P2B[6] rotavirus Gottfried strain efficiently with titers ranging from identical to eight-fold less than to the homotypic human P2A[6] strains. Antibodies to Gottfried VP4, however, neutralized human P2A[6] or P?[6] rotavirus strains recovered from asymptomatic or symptomatic infections 8- to 16-fold less efficiently than the homologous Gottfried strain. Thus, because of the more efficient neutralization, the established human P2A[6] rotavirus strains could be considered as prime strains relative to the Gottfried strain. Thus, this is in accord with a designation of the Gottfried virus as a subtype (P2B[6]) strain as suggested by Li and Gorziglia (1993). However, as noted earlier, our neutralization results were not in agreement with this latter study, which found significant neutralization of neonatal P2A strains by antiserum to a Gottfried virus recombinant VP4 but did not find significant neutralization of Gottfried virus by antiserum to a P2A neonatal strain recombinant VP4.

Previously, Chen et al. reported that a cross-reactive, neutralizing VP4-specific epitope was affected by specific interactions between the VP4 and VP7 of selected reassortants (Chen et al., 1992). More recently, Pesavento et al. reported that the subtle alterations in VP4 conformation in certain reassortants were responsible for such altered VP4 phenotype (Pesavento et al., 2003). However, the effect of such alterations in VP4 conformation in reassortants on the expression of anti-VP4 neutralizing antibodies as a whole has never been studied. In this study, we analyzed whether any qualitative and quantitative differences could be detected in anti-M37 VP4 neutralizing antibodies raised against reassortants M37 × DS-1 (P2A[6],G2) or M37 × UK (P2A[6],G6) and showed that there were no appreciable

differences in neutralizing capabilities between the two anti-M37 VP4 antibodies.

As noted earlier, two of the endemic nursery P[6] rotavirus isolates (Venezuelan M37 strain or Australian RV3 strain) have been developed independently by us and others as candidate vaccines and undergone phase I to a limited phase II clinical trials (Barnes et al., 1997, 2002; Flores et al., 1990; Midthun et al., 1991; Perez-Schael et al., 1994; Vesikari et al., 1991). Both M37 (P2A[6],G1) and RV3 (P2A[6], G3) vaccines have been shown to be satisfactorily attenuated and immunogenic in infants. Studies with the M37 have been discontinued; however, the RV3 candidate is under study as stated earlier.

Development of a porcine rotavirus strain Gottfried (P2B[6],G4)-based reassortant vaccine, which incorporates human rotavirus VP7 (G1, G2, G3 or G9) or VP4 (P1A[8] or P1B[4]) genes of epidemiologic importance, may represent an interesting approach because it could provide (i) an attenuation phenotype of a porcine rotavirus in humans and (ii) antigenic coverage not only for VP7 and VP4 serotypes of epidemiologic importance but also for P2A[6] serotype, which in some parts of the world appears to be of clinical significance.

Materials and methods

Rotavirus strains, cell cultures, virus titration, neutralization assay, and hyperimmune antiserum

Table 1 summarizes the rotavirus strains used in this study. Four P2A[6] rotavirus strains recovered from asymptomatic neonates were M37 from Venezuela, 1076 from Sweden, McN from Australia, and ST3 from the United Kingdom. Four P2A[6] or P?[6] rotavirus strains derived from symptomatic infections were MW23 from Malawi, US1205 from the United States, INL1 from India, and R143 from Brazil. P and G types and year of collection of each of the eight P[6] strains are shown in Table 1. Primary cultures of African green monkey kidney (AGMK) cells (Whittaker Bioproducts, Walkersville, MD) or an established monkey kidney MA104 cell line were used for genetic reassortment, plaque purification, and virus amplification. The MA104 cell line was used for virus titration and plaque reduction neutralization (PRN) assay. Eagle's minimum essential medium supplemented with 0.5 μg/ml trypsin (Sigma γ-irradiated trypsin, Sigma Chemical, St. Louis, MO) and antibiotics were used as maintenance medium. Hyperimmune antiserum to each of the reassortants was raised in specific pathogen-free guinea pigs (National Cancer Institute, Frederick, MD), which were free of rotavirus neutralizing antibodies (titer < 1:20 versus M37) as determined by PRN assay. Rotavirus immunogens were prepared as previously described (Wyatt et al., 1982).

Generation, identification, and characterization of single gene substitution rotavirus reassortants

Roller tube cultures of primary AGMK or MA104 cells were coinfected at a multiplicity of infection of approximately 1 with (i) the M37 virus and the DS-1 virus, (ii) the M37 virus and the UK virus, (iii) the ST3 virus and the DS-1 virus, (iv) the MW23 virus and the UK virus, (v) the US1205 virus and the UK virus, or (vi) the Gottfried virus and the DS-1 virus. When approximately 75% of the infected cells exhibited cytopathic effects, the cultures were frozen and thawed once and the lysate was plated onto primary AGMK or MA104 cells in a six-well plate in the presence of (i) G1-specific VP7 neutralizing monoclonal antibody (N-mAb) 2C9 (Shaw et al., 1985) for selection of the desired M37 $\times$ DS-1 (P2A[6],G2) or M37 $\times$ UK (P2A[6],G6), (ii) G4-specific N-mAb ST-2G7 (Taniguchi et al., 1987) for selection of the desired ST3 $\times$ DS-1 (P2A[6],G2) or Gottfried $\times$ DS-1 (P2B[6],G2), (iii) guinea pig hyperimmune antiserum to human rotavirus 69M strain (P4[10],G8) for selection of the desired MW23 $\times$ UK (P2A[6],G6), or (iv) guinea pig hyperimmune antiserum to human rotavirus WI61 strain (P1A[8],G9) for selection of the desired US1205 $\times$ UK (P2A[6],G6). Each of the desired reassortants was selected and identified and then plaque purified three times. Each plaque-purified reassortant was examined for G serotype by enzyme-linked immunosorbent assay using type-specific VP7 N-mAbs including G6-specific UK/7 (Snodgrass et al., 1990) as described previously (Green et al., 1990). The origin of genes of each reassortant was identified by polyacrylamide gel electrophoresis (PAGE) of its genomic RNAs. The origin of certain genes which was not able to be determined with certainty by PAGE was studied further by constant denaturant gel electrophoresis (CDGE) as previously described (Jones et al., 2003). Hyperimmune guinea pig antiserum to each reassortant was tested for antibodies to selected human and animal rotavirus strains by 60% PRN assay (Hoshino et al., 1998).

References

Albrey, M.B., Murphy, A.M., 1976. Rotaviruses and acute gastroenteritis of infants and children. Med. J. Aust. 1, 82–85.

Barnes, G.L., Lund, J.S., Adams, L., Mora, A., Mitchell, S.V., Caples, A., Bishop, R.F., 1997. Phase I trial of a candidate rotavirus vaccine (RV3) derived from a human neonate. J. Paediatr. Child Health 33, 300–304.

Barnes, G.L., Lund, J.S., Mitchell, S.V., De Bruyn, L., Piggford, L., Smith, A.L., Furmedge, J., Masendycz, P.J., Bugg, H.C., Bogdanovic-Sakran, N., Carlin, J.B., Bishop, R.F., 2002. Early phase II trial of human rotavirus vaccine candidate RV3. Vaccine 20, 2950–2956.

Bishop, R.F., Barnes, G.L., Cipriani, E., Lund, J.S., 1983. Clinical immunity after neonatal rotavirus infection. A prospective longitudinal study in young children. N. Engl. J. Med. 309, 72–76.

Bishop, R.F., Hewstone, A.S., Davidson, G.P., Townley, R.R.W., Holmes, I.H., Ruck, B.J., 1976. An epidemic of diarrhoea in human neonates involving a reovirus-like agent and 'enteropathogenic' serotypes of *Escherichia coli*. J. Clin. Pathol. 29, 46–49.

Bohl, E.H., Theil, K.W., Saif, L.J., 1984. Isolation and serotyping of porcine rotaviruses and antigenic comparison with other rotaviruses. J. Clin. Microbiol. 19, 105–111.

Bresee, J.S., Glass, R.I., Ivanoff, B., Gentsch, J.R., 1999. Current status and future priorities for rotavirus vaccine development, evaluation and implementation in developing countries. Vaccine 17, 2207–2222.

Bridger, J.C., Tauscher, G.I., Desselberger, U., 1998. Viral determinants of rotavirus pathogenicity in pigs: evidence that the fourth gene of a porcine rotavirus confers diarrhea in the homologous host. J. Virol. 72, 6929–6931.

Broome, R.L., Vo, P.T., Ward, R.L., Clark, H.F., Greenberg, H.B., 1993. Murine rotavirus genes encoding outer capsid proteins VP4 and VP7 are not major determinants of host range restriction and virulence. J. Virol. 67, 2448–2455.

Bryden, A.S., Thouless, M.E., Hall, C.J., Flewett, T.H., Wharton, P.M., Mathew, P.M., Craig, I., 1982. Rotavirus infections in a special-care baby unit. J. Infect. 4, 43–48.

Chen, D., Estes, M.K., Ramig, R.F., 1992. Specific interactions between rotavirus outer capsid proteins VP4 and VP7 determine expression of a cross-reactive, neutralizing VP4-specific epitope. J. Virol. 66, 432–439.

Chrystie, I.L., Totterdell, B., Baker, M.J., Scopes, J.W., Banatvala, J.E. 1975. Rotavirus infection in maternity unit. Lancet ii, 79.

Cicirello, H.G., Das, B.K., Gupta, A., Bhan, M.K., Gentsch, J.R., Kumar, R., Glass, R.I., 1994. High prevalence of rotavirus infection among neonates born at hospitals in Delhi, India: predisposition of newborns for infection with unusual rotavirus. Pediatr. Infect. Dis. J. 13, 720–724.

Cunliffe, N.A., Bresee, J.S., Gentsch, J.R., Glass, R.I., Hart, C.A., 2001. The expanding diversity of rotaviruses. Lancet 359, 640–642.

Cunliffe, N.A., Gentsch, J.R., Kirkwood, C.D., Gondwe, J.S., Dove, W., Nakagomi, O., Nakagomi, T., Hoshino, Y., Bresee, J.S., Glass, R.I., Molyneux, M.E., Hart, C.A., 2000. Molecular and serologic characterization of novel serotype G8 human rotavirus strains detected in Blantyre, Malawi. Virology 274, 309–320.

Das, B.K., Gentsch, J.R., Cicirello, H.G., Woods, P.A., Gupta, A., Ramachandran, M., Kumar, R., Bhan, M.K., Glass, R.I., 1994. Characterization of rotavirus strains from newborns in New Delhi, India. J. Clin. Microbiol. 32, 1820–1822.

Flores, J., Midthun, K., Hoshino, Y, Green, K., Gorziglia, M., Kapikian, A.Z., Chanock, R.M., 1986. Conservation of the fourth gene among rotaviruses recovered from asymptomatic newborn infants and its possible role in attenuation. J. Virol. 60, 972–979.

Flores, J., Perez-Schael, I., Blanco, M., White, L., Garcia, D., Vilar, M., Cunto, W., Gonzalez, R., Urbina, C., Boher, J., Mendez, M., Kapikian, A.Z., 1990. Comparison of reactogenicity and antigenicity of M37 rotavirus vaccine and rhesus-rotavirus-based quadrivalent vaccine. Lancet 336, 330–334.

Garbag-Chenon, A., Brussieux, J., Boisivon, A., Nicolas, J.C., Bricout, F., 1985. Epidemiology of human rotaviruses in a maternity unit as studied by electrophoresis of genomic RNA. Eur. J. Epidemiol. 1, 33–36.

Gentsch, J.R., Woods, P.A., Ramachandran, M., Das, B.K., Leite, J.P., Alfieri, A., Kumar, R., Bhan, M.K., Glass, R.I., 1996. Review of G and P typing results from a global collection of rotavirus strains: implications for vaccine development. J. Infect. Dis. 174 (Suppl. 1), S30–S36.

Glass, R.I., Gentsch, J., Smith, J.C., 1994. Rotavirus vaccines: success by reassortment? Science 265, 1389–1391.

Gorziglia, M., Green, K., Nishikawa, K, Taniguchi, K., Jones, R., Kapikian, A.Z., Chanock, R.M., 1988. Sequence of the fourth gene of human rotaviruses recovered from asymptomatic or symptomatic infections. J. Virol. 62, 2978–2984.

Gorziglia, M., Larralde, G., Kapikian, A.Z., Chanock, R.M., 1990. Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. Proc. Natil. Acad. Sci. USA 87, 7155–7159.

Gorziglia, M., Nishikawa, K., Hoshino, Y., Taniguchi, K., 1990. Similarity of the outer capsid protein VP4 of the Gottfried strain of porcine rotavirus to that of asymptomatic human rotavirus trains. J. Virol. 64, 414–418.

Green, K.Y., James Jr., H.D., Kapikian, A.Z., 1990. Evaluation of three panels of monoclonal antibodies for the identification of human rotavirus VP7 serotype by ELISA. Bull. WHO 68, 601–610.

Grillner, L., Broberger, U., Chrystie, I., Ransjo, U., 1985. Rotavirus infections in newborns: an epidemiological and clinical study. Scand. J. Infect. Dis. 17, 349–355.

Haffejee, I.E., 1991. Neonatal rotavirus infections. Rev. Infect. Dis. 13, 957–962.

Haffejee, I.E., Moosa, A., Windsor, I., 1990. Circulating and breast-milk anti-rotaviral antibodies and neonatal rotavirus infections: a maternal-neonatal study. Ann. Trop. Paediatr. 10, 3–14.

Hoshino, Y., Kapikian, A.Z., 1996. Classification of rotavirus VP4 and VP7 serotypes. Arch Virol [Suppl] 12, 99–111.

Hoshino, Y., Kapikian, A.Z., 2000. Rotavirus serotypes: classification and importance in epidemiology, immunity, and vaccine development. J. Health Popul. Nutr. 18, 5–14.

Hoshino, Y., Jones, R.W., Kapikian, A.Z., 1998. Serotypic characterization of outer capsid spike protein VP4 of vervet monkey rotavirus SA11 strain. Arch. Virol. 143, 1233–1244.

Hoshino, Y., Saif, L.J., Kang, S-Y., Sereno, M.M., Chen, W-K., Kapikian, A.Z., 1995. Identification of group A rotavirus genes associated with virulence of a porcine rotavirus and host range restriction of a human rotavirus in the gnotobiotic piglet model. Virology 209, 274–280.

Hoshino, Y., Wyatt, R.G., Flores, J., Midthun, K., Kapikian, A.Z., 1985. Serotypic characterization of rotaviruses derived from asymptomatic human neonatal infections. J. Clin. Microbiol. 21, 425–430.

Jayashree, S., Bhan, M.K., Raj, P., Kumar, R., Svensson, L., Stintzing, G., Bhandari, N., 1988. Neonatal rotavirus infection and its relation to cord blood antibodies. Scand. J. Infect. Dis. 20, 249–253.

Jones, R.W., Ross, J., Hoshino, Y., 2003. Identification of parental origin of cognate dsRNA genome segment(s) of rotavirus reassortants by constant denaturant gel electrophoresis. J. Clin. Virol. 26, 347–354.

Kapikian, A.Z., Hoshino, Y., Chanock, R.M., 2001. Rotaviruses. in:Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology, fourth ed. Lippincott Williams & Wilkins, Philadelphia, pp. 1787–1833.

Kilgore, P.E., Unicomb, L.E., Gentsch, J.R., Albert, M.J., McElroy, C.A., Glass, R.I., 1996. Neonatal rotavirus infection in Bangladesh: strain characterization and risk factors for nosocomial infection. Pediatr. Infect. Dis. J. 15, 672–677.

Kirkwood, C.D., Coulson, B.S., Bishop, R.F., 1996. G3P2 rotaviruses causing diarrhoeal disease in neonates differ in VP4, VP7 and NSP4 sequence from G3P2 strains causing asymptomatic neonatal infection. Arch. Virol. 141, 1661–1676.

Kirkwood, C.D., Gentsch, J.R., Hoshino, Y., Clark, H.F., Glass, R.I., 1999. Genetic and antigenic characterization of a serotype P[6]G9 human rotavirus strain isolated in the United States. Virology 256, 45–53.

Koshimura, Y., Nakagomi, T., Nakagomi, O., 2000. The relative frequencies of G serotypes of rotaviruses recovered from hospitalized children with diarrhea: a 10-year survey (1987–1996) in Japan with a review of globally collected data. Microbiol. Immunol. 44, 499–510.

Lee, C.-N., Lin, C-C., Kao, C-L., Zao, C-L., Shih, M-C., Chen, H-N., 2001. Genetic characterization of the rotaviruses associated with a nursery outbreak. J. Med. Virol. 63, 311–320.

Lee, C-N., Wang, Y-L., Kao, C-L., Zao, C-L., Chen, H-N., 2000. NSP4 gene analysis of rotaviruses recovered from infected children with and without diarrhea. J. Clin. Microbiol. 38, 4471–4477.

Li, B., Gorziglia, M., 1993. VP4 serotype of the Gottfried strain of porcine rotavirus. J. Clin. Microbiol. 31, 3075–3077.

Linhares, A.C., Mascarenhas, J.D.P., Gusmao, R.H.P., Gabbay, Y.B., Fialho, A.M., Leite, J.P.G., 2002. Neonatal rotavirus infection in Belem, Northern Brazil: nosocomial transmission of a P[6] strain. J. Med. Virol. 67, 418–426.

Madeley, C.R., Cosgrove, B.P., Bell, E.J., 1978. Stool viruses in babies in Glasgow. II. Investigation of normal newborns in hospital. J. Hyg. 81, 285–294.

Midthun, K., Halsey, N.A., Jett-Goheen, M., Clements, M.L., Steinhoff, M., King, J.C., Karron, R., Wilson, M., Burns, B., Perkis, V., Samorodin, R., Kapikian, A.Z., 1991. Safety and immunogenicity of human rotavirus vaccine strain M37 in adults, children, and infants. J. Infect. Dis. 164, 792–796.

Miller, M.A., McCann, L., 2000. Policy analysis of the use of hepatitis B, *Haemophilus influenzae* type B-, and rotavirus vaccines in national immunization schedules. Health Promot. 9, 19–35.

Murphy, A.M., Albrey, M.B., Hay, P.J., 1975. Rotavirus infection in neonates. Lancet ii, 452–453.

Oelofsen, M.J., Venter, E.H., Smith, M.S., 1985. Nosocomial rotavirus infections in a South African hospital nursery for white infants. S. Afr. Med. J. 68, 394–396.

Offit, P.A., Blavat, G., Greenberg, H.B., Clark, H.F., 1986. Molecular basis of rotavirus virulence: role of gene segment 4. J. Virol. 57, 46–49.

Pager, C.T., Alexander, J.J., Steele, A.D., 2000. South African G4P[6] asymptomatic and symptomatic neonatal rotavirus strains differ in their NSP4, VP8*, and VP7 genes. J. Med. Virol. 62, 208–216.

Palombo, E.A., Bishop, R.F., 1994. Genetic analysis of NSP1 genes of human rotaviruses isolated from neonates with asymptomatic infection. J. Gen. Virol. 75, 3635–3639.

Perez-Schael, I., Blanco, M., Garcia, D., White, L., Alfonzo, E., Crespo, I., Cunto, W., Pittman, A.L., Kapikian, A.Z., Flores, J., 1994. Evaluation of the antigenicity and reactogenicity of varying formulations of the rhesus rotavirus-based quadrivalent and the M37 rotavirus vaccine candidates. J. Med. Virol. 42, 330–337.

Perez-Schael, I., Daoud, G., White, L., Urbina, G., Daoud, N., Perez, M., Flores, J., 1984. Rotavirus shedding by newborn children. J. Med. Virol. 14, 127–136.

Pesavento, J.B., Billingsley, A.M., Roberts, E.J., Ramig, R.F., Prasad, B.V.V., 2003. Structures of rotavirus reassortants demonstrate correlation of altered conformation of the VP4 spike and expression of unexpected VP4-associated phenotypes. J. Virol. 77, 3291–3296.

Ramachandran, M., Gentsch, J.R., Parashar, U.D., Jin, S., Woods, P.A., Holmes, J.L., Kirkwood, C.D., Bishop, R.F., Greenberg, H.B., Urasawa, S., Gerna, G., Coulson, B.S., Taniguchi, K., Bresee, J.S., Glass, R.I., The National Rotavirus Strain Surveillance System Collaborating Laboratories, 1998. Detection and characterization of novel rotavirus strains in the United States. J. Clin. Microbiol. 36, 3223–3229.

Ramachandran, M., Kirkwood, C.D., Unicomb, L., Cunliffe, N.A., Ward, R.L., Bhan, M.K., Clark, H.F., Glass, R.I., Gentsch, J.R., 2000. Molecular characterization of serotype G9 rotavirus strains from a global collection. Virology 278, 436–444.

Santos, N., Gouvea, V., Timenetsky, M.C., Clark, H.F., Riepenhoff-Talty, M., Garbarg-Chenon, A., 1994. Comparative analysis of VP8* se-

quences from rotaviruses possessing M37-like VP4 recovered from children with and without diarrhoea. J. Gen. Virol. 75, 1775–1780.

Santos, N., Volotao, E.M., Soares, C.C., Albuquerque, M.C.M., Da Silva, F.M., De Carvalho, T.R.B., Pereira, C.F.A., Chizhikov, V., Hoshino, Y., 2001. Rotavirus strains bearing genotype G9 or P[9] recovered from Brazilian children with diarrhea from 1997 to 1999. J. Clin. Microbiol. 39, 1157–1160.

Shaw, R.D., Stoner-Ma, D.L., Estes, M.K., Greenberg, H.B., 1985. Specific enzyme-linked immunoassay for rotavirus serotypes 1 and 3. J. Clin. Microbiol. 22, 286–291.

Snodgrass, D.R., Fitzgerald, T., Campbell, I., Scott, F.M., Browning, G.F., Miller, D.L., Herring, A.J., Greenberg, H.B., 1990. Rotavirus serotypes 6 and 10 predominate in cattle. J. Clin. Microbiol. 28, 504–507.

Spencer, E., Araya, M., Sandino, A.M., Pacheco, I., 1988. Faecal excretion of rotavirus and other enteropathogens in newborns of the high and low socio-economic stratum in Santiago, Chile. Epidemiol. Infect. 101, 425–436.

Steele, A.D., Alexander, J.J., 1987. The relative frequency of subgroup I and II rotaviruses in Black infants in South Africa. J. Med. Virol. 24, 321–327.

Steele, D., Reynecke, E., de Beer, M., Bos, P., Smuts, I., 2002. Characterization of rotavirus infection in a hospital neonatal unit in Pretoria, South Africa. J. Trop. Pediatr. 48, 167–171.

Sukumaran, M., Gowda, K., Maiya, P.P., Srinivas, T.P., Kumar, M.S., Aijaz, S., Reddy, R.R., Padilla, L., Greenberg, H.B., Rao, C.D., 1992. Exclusive asymptomatic neonatal infections by human rotavirus strains having subgroup I specificity and "long" RNA electropherotype. Arch. Virol. 126, 239–251.

Tam, J.S., Zheng, B.J., Lo, S.K., Yeung, C.Y., Lo, M., Ng, M.H., 1990. Distinct populations of rotaviruses circulating among neonates and older infants. J. Clin. Microbiol. 28, 1033–1038.

Taniguchi, K., Urasawa, T., Morita, Y., Greenberg, H.B., Urasawa, S., 1987. Direct serotyping of human rotavirus in stools by an enzyme-linked immunosorbent assay using serotype 1-, 2-, 3-, or 4-specific monoclonal antibodies to VP7. J. Infect. Dis. 155, 1159–1166.

Tietzova, J., Petovicova, A., Pazdiora, P., 1995. Characterization of human rotaviruses isolated from symptomatic and asymptomatic infections. Acta Virol. 39, 211–214.

Totterdell, B.M., Chrystie, I.L., Banatvala, J.E., 1976. Rotavirus infections in a maternity unit. Arch. Dis. Childhood 51, 924–928.

Tufvesson, B., Polberger, S., Svanberg, L., Sveger, T., 1986. A prospective study of rotavirus infections in neonatal and maternity wards. Acta Paediatr. Scand. 75, 211–215.

Vesikari, T., Ruuska, R., Koivu, H.P., Green, K.Y., Flores, J., Kapikian, A.Z., 1991. Evaluation of the M37 human rotavirus vaccine in 2- to 6-month-old infants. Pediatr. Infect. Dis. J. 10, 912–917.

Woode, G.N., Jones, J., Bridger, J., 1975. Levels of colostral antibodies against neonatal calf diarrhoea virus. Vet. Rec. 97, 148–149.

Wyatt, R.G., Greenberg, H.B., James, W.D., Pittman, A.L., Kalica, A.R., Flores, J., Chanock, R.M., Kapikian, A.Z., 1982. Definition of human rotavirus serotypes by plaque reduction assay. Infect. Immunol. 37, 110–115.

Wyatt, R.G., James Jr., H.D., Pittman, A.L., Hoshino, Y., Greenberg, H.B., Kalica, A.R., Flores, J., Kapikian, A.Z., 1983. Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. J. Clin. Microbiol. 18, 310–317.